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Quasispecies Nature of Hepatitis C Virus (HCV) in Patients With Chronic Hepatitis C With Mixed HCV Subtypes

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The quasispecies nature of hepatitis C virus (HCV) in patients with mixed HCV subtype infection was compared with that in patients with single HCV subtype infection. The number of HCV quasispecies was compared between 35 patients with mixed HCV subtype infection and 83 patients with single subtype infection. Subtype was determined by primers deduced from the core region and by line probe assay respectively. The number of quasispecies was evaluated by polymerase chain reaction amplification of hypervariable region 1 and by fluorescence single-strand conformation polymorphism analysis. There was no difference in clinical background between patients with mixed subtype infection and patients with single subtype infection. The number of quasispecies in patients with multiple subtype HCV infection was larger than in patients with single subtype HCV infection. The immunologic environments which allow the coexistence of more HCV quasispecies in patients with multiple HCV subtype infection differs from that in patients with single HCV subtype infection. ***J. Med. Virol. 54:80–85, 1998.***

KEY WORDS: hepatitis C virus; mixed subtypes; quasispecies; immunologic environment

INTRODUCTION

Hepatitis C virus (HCV) was cloned molecularly in 1989 [Choo et al., 1989], and different genotypes and subtypes have been characterized [Okamoto et al., 1992; Simmonds et al., 1993; McOmish et al., 1993]. Further study has revealed that HCV isolates consist of heterogeneous mixtures of genetically different, but closely related, variants even in patients with single HCV subtype infection [Oshima et al., 1991; Martell et al., 1992; Murakawa et al., 1992]. These variants are termed 'quasispecies' and their presence has been demonstrated recently to be relevant to persistent infection [Weiner et al., 1992], clinical features [Zonaro et al., 1994; Enomoto et al., 1994a], viral titers [Zonaro et al., 1994], and disease progression [Honda et al., 1994; Koizumi et al., 1995].

Mixed subtype HCV infections have been observed, although the prevalence of patients with mixed HCV subtype infection has been reported to be low [Dusheiko et al., 1994; McOmish et al., 1994; Mahaney et al., 1994; Tanaka et al., 1995]. In patients with mixed subtypes there are at least two HCV subtypes and they can have even more HCV quasispecies. Do more HCV quasispecies exist in patients with mixed HCV subtype infection than in patients with single HCV subtype infection? Conversely, are there similar numbers of HCV quasispecies in patients with single HCV subtype infection and those with mixed subtype infection?

To address these questions, the quasispecies nature of HCV in patients with single subtype HCV infection was compared with mixed subtype HCV infection.

PATIENTS AND METHODS

Patients

Subtyping of HCV RNA was carried out on sera from 382 patients with chronic hepatitis C who lived in the Tokai area in the center of Honshu Island in Japan and who had been followed for more than 3 years at Nagoya University Hospital, Sakashita Municipal Hospital, or Ogaki Municipal Hospital, all in the Tokai region, as outpatients. They consisted of 83 patients with hemophilia or other coagulation disorders (coagulopathy

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TABLE I. Combinations of Subtypes in Patients with HCV Infection by Mixed Subtypes

Combination of subtypes	Number of patients
1a + 1b	11
1a + 2b	1
1b + 2a	16
1b + 3a	4
1a + 1b + 2a	1
1a + 1b + 3a	1
1a + 1b + 2b + 3a	1
Total	35

group) [Isobe et al., 1995], 20 patients who were presumed to be infected via blood transfusion (posttransfusion group), 122 patients who lived in an isolated area where hepatitis C was endemic and had practiced such traditional remedies as acupuncture (community-acquired infection group) [Kiyosawa et al., 1994; Yamada et al., 1994], and 157 patients without a recognized mode of transmission (sporadic group). The presence of HCV was proved in all patients by both HCV antibody assay (2nd generation, Dinabot; Tokyo, Japan) and HCV RNA detection by the nested reverse transcription polymerase chain reaction (RT-PCR) method [Okamoto et al., 1990]. None had a history of antiviral therapy such as interferon administration before sampling of serum. Subtyping of HCV RNA was undertaken by PCR amplification of core gene sequences with genotype-specific primers, as described by Okamoto et al. [1992 & 1993], and by using the line probe assay [Stuyver et al., 1993] respectively. The subtypes were classified as 1a, 1b, 2a, 2b, and 3a according to Simmonds' nomenclature [Simmonds et al., 1994]. Subtypes were identified without discrepancy between subtype by PCR with genotype-specific primers and that by line probe assay in 346 of 382 patients. Mixed subtypes were detected in 35 patients, and 311 patients had a single HCV subtype. The combinations of subtypes in patients with HCV infection by mixed subtypes are shown in Table I. HCV quasispecies of all 35 patients with a mixture of HCV subtypes were compared with that of 83 patients with a single HCV subtype who had been selected from 311 patients with a single HCV subtype to match the proportion of mode of transmission with that of patients with mixed HCV subtypes.

Analysis of HCV Quasispecies by Fluorescence Single-Strand Conformation Polymorphism (SSCP)

Hypervariable region 1 (HVR1) of HCV was amplified by PCR using a newly developed primer set [Toyoda et al., 1997]. This primer set was designed by analyzing 128 reported variations of the HCV RNA genome, so that the primer sequences were more than 80% homologous to all described isolates. The primer sequences were as follows: TGGGACACATGATGATGAACTGGT (sense primer for the first PCR), TACTACTCCATGGTGGGAGACTGGGC (sense primer for the second PCR), GATGTGCCAGCTGCCATTGG (antisense primer for the second PCR), CGGTGCTGTTTATGTGCCAACTGCC (antisense primer for the first PCR and reverse transcription). The antisense primer for the second PCR was labeled with fluorescein isothiocyanate (FITC) during synthesis.

The number of quasispecies was determined by fluorescence SSCP analysis using an automated sequencer [Orita et al., 1989; Enomoto et al., 1994b; Makino et al., 1992; Kinoshita et al., 1994]. PCR products were denatured for 5 min at 95°C in formamide dye (Pharmacia Biotech; Uppsala, Sweden), and electrophoresed on an ALFII DNA Sequencer (Pharmacia Biotech; Uppsala, Sweden). Detection of HCV quasispecies was carried out using the Fragment Manager (Pharmacia Biotech; Uppsala, Sweden) software system.

Quantitation of HCV RNA

Serum HCV RNA concentrations were measured by branched DNA (bDNA) probe assay [Lau et al., 1993; Urdea, 1993]. A branched DNA signal-amplification assay kit (Quantiplex™ HCV-RNA, version 2.0, Chiron Corporation, Emeryville, CA) was used [Detmer et al., 1996].

Statistical Analysis

Results are expressed as mean ± SD. Differences in proportions were analyzed by the chi-square test. Mean quantitative values were compared by Student's *t*-test. Nonparametric data were compared using the Mann–Whitney U test. All *P* values were two-tailed and a level of <0.05 was accepted as statistically significant. The entire protocol was approved by the hospital ethics committee and carried out in compliance with the Helsinki declaration.

RESULTS

Characteristics and HCV RNA Concentration of Patients with Single HCV Subtype and of Patients with Mixed HCV Subtypes

Table II shows the characteristics of patients with single or mixed HCV subtype infection. Patients with coagulopathy or community-acquired infection were predominant [Isobe et al., 1995; Yamada et al., 1994]. There were no significant differences in background between these two groups.

HCV RNA was undetectable by bDNA probe assay in 8 of 83 patients with a single HCV subtype and in 2 of 35 patients with mixed HCV subtypes. In the other patients, HCV RNA concentrations were similar between patients with a single HCV subtype ($6.07 \pm 7.21 \times 10^6$ eq/mL) and in those with mixed HCV subtypes ($5.73 \pm 7.63 \times 10^6$ eq/mL) ($P = 0.7237$, Fig. 1).

Number of Quasispecies in Patients with a Single HCV Subtype and Patients with Mixed HCV Subtypes

HVR1 were amplified by RT-PCR in 77 patients with a single HCV subtype and in 31 patients with mixed subtypes. The number of quasispecies detected by fluo-

TABLE II. Characteristics of Patients with HCV Infection by Single or Mixed Subtypes*

	Single HCV subtype	Mixed HCV subtypes
Number	83	35
Male:Female	62:21	25:10
Mean age	43.7 ± 13.4	41.1 ± 14.1
Mode of transmission		
Sporadic	15	6
Community-acquired infection	28	11
Coagulopathy	40	18
Duration of HCV carriage [Median (years), range]	4.23 (0.2–20)	3.94 (0.2–28)
Biochemistry (Mean ± SD)		
Alanine aminotransferase (IU/L)	73.6 ± 54.0	107.8 ± 65.6
Asparate aminotransferase (IU/L)	55.6 ± 37.4	77.6 ± 47.6
Albumin (mg/L)	4.30 ± 0.37	4.27 ± 0.30
Globulin (mg/L)	3.30 ± 0.56	3.28 ± 0.63
Alkaline phosphatase (IU/L)	168.2 ± 67.7	162.9 ± 74.1
γ-glutamyl transpeptidase (mU/mL)	48.8 ± 48.3	79.0 ± 88.1
Total bilirubin (mg/dL)	0.77 ± 0.37	0.77 ± 0.26

*HCV: Hepatitis C virus; PCR: Polymerase chain reaction.

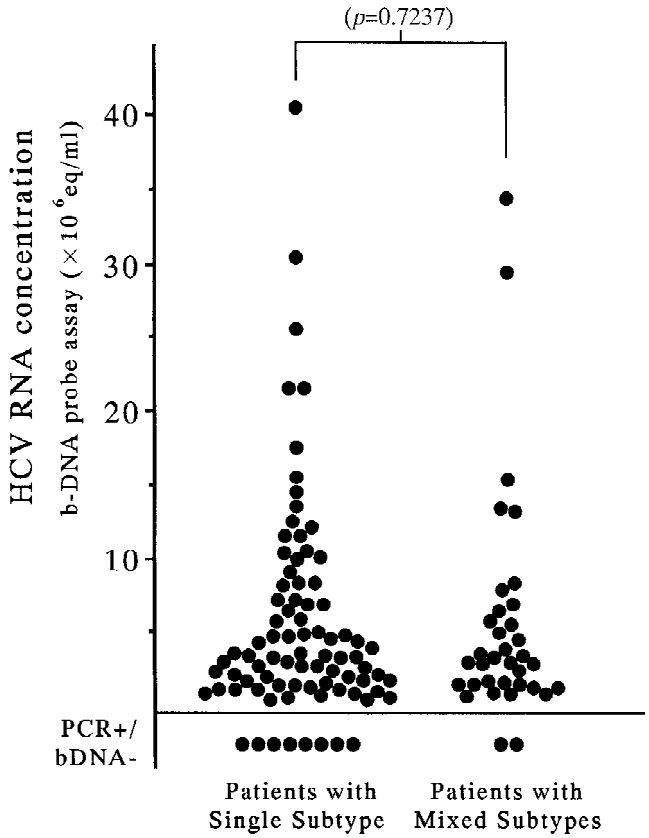

Fig. 1. HCV RNA concentration in patients with single and mixed HCV subtype infection. There was no significant difference in HCV RNA concentration between the two groups. HCV: Hepatitis C virus.

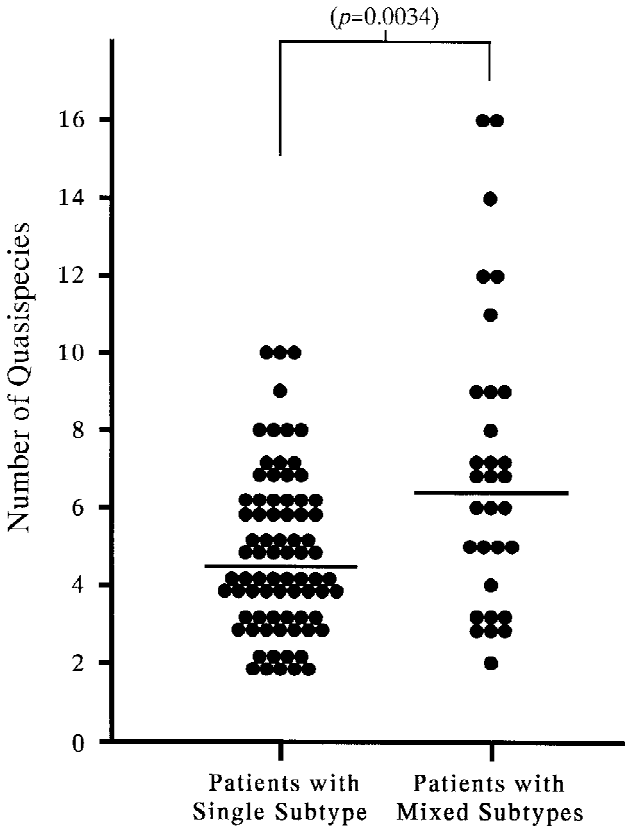

Fig. 2. Comparison of the number of HCV quasispecies in the serum of patients with single and mixed HCV subtype infection. The number of quasispecies was significantly larger in patients with mixed subtype infection than in patients with single subtype infection ($P = 0.0034$). HCV: Hepatitis C virus.

rescence-SSCP analysis in patients with single HCV subtype infection and patients with mixed subtype infection is shown in Figure 2. The number of HCV quasispecies in the serum of patients with mixed HCV subtypes was significantly higher than in patients with a single subtype (7.10 ± 3.82 in patients with a mixture of subtypes vs. 4.83 ± 2.04 in patients with a single subtype, $P = 0.0034$). The number of HCV quasispecies per subtype (number of quasispecies/number of sub-

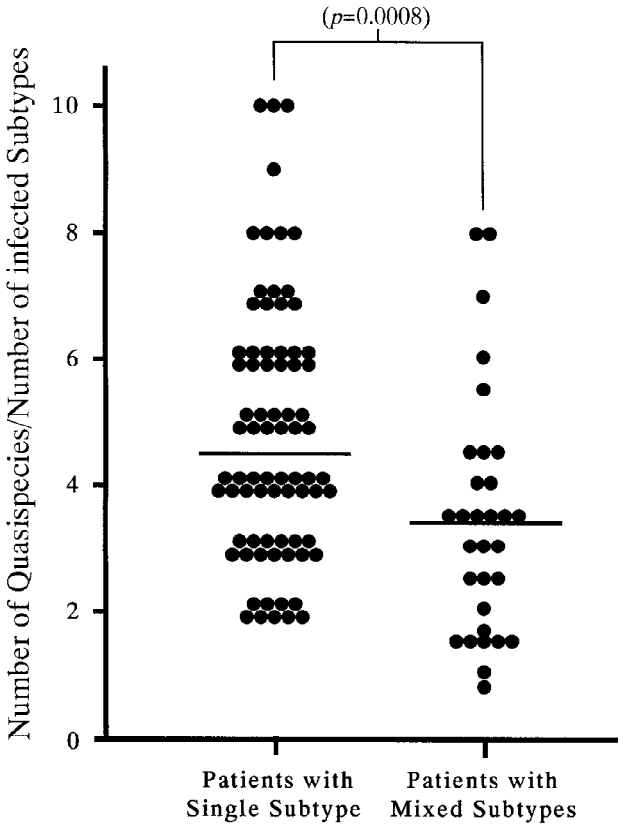

Fig. 3. Comparison of the number of HCV quasispecies in patients with single HCV subtype infection and the number of quasispecies divided by the number of infected subtypes in patients with mixed HCV subtype infection. The number of quasispecies in patients with single subtype infection was significantly larger than the number of quasispecies/the number of subtypes in patients with mixed subtype infection ($P = 0.0008$). HCV: Hepatitis C virus.

types) in patients was 3.43 ± 1.91, and significantly lower than in patients with a single subtype ($P = 0.0008$, Fig. 3).

DISCUSSION

HCV infections with a mixture of subtypes are observed rarely, with a reported prevalence of under 5% [Dusheiko et al., 1994; McOmish et al., 1994; Mahaney et al., 1994; Tanaka et al., 1995; Chan et al., 1995; Chayama et al., 1993; Prati et al., 1996]. Coexistence of HCV multiple subtypes can be produced by multiple infections with HCV strains of different subtypes. However, the superinfection of a HCV-infected individual with another HCV subtype does not always result in coinfection with HCV of mixed subtypes. Infection by HCV of another subtype superimposed on previously existing HCV infection with subsequent exclusion of superinfected HCV strains have been observed in human and experimentally infected chimpanzees [Kao et al., 1993; Okamoto et al., 1994]. Therefore, some patients must have characteristics permitting infection by multiple HCV subtypes, though their nature is unknown.

HCV isolates in a given patient consist of a mixture of quasispecies. The quasispecies of HCV is considered to be produced as a result of spontaneous mutation of the HCV genome in the attempt to maintain a persistent infection despite the host's immune defenses [Weiner et al., 1992], and HCV quasispecies to which specific antibodies were produced were reported to disappear from serum [Kato et al., 1993] and the increase of the number of quasispecies may be suppressed by this. In the present study the number of HCV quasispecies detected by SSCP analysis of PCR amplified products of HVR1 was induced because HVR1 is the most sensitive region for distinguishing different HCV quasispecies.

On comparing quasispecies between patients with single HCV subtype infection and those with mixed infections, larger numbers of quasispecies were detected in patients with mixed subtype infections, though the number of quasispecies was not linear function of the number of infected subtypes. This indicated that there may be more quasispecies in patients in whom multiple subtypes of HCV can coexist than in patients with single HCV subtype infection, and suggests that there are differences in immunologic environment which allow the coexistence of more HCV quasispecies in patients with multiple HCV subtypes.

A high prevalence of infection with a mixture of HCV subtypes has been reported in patients who are at high risk of exposure to HCV, such as hemophiliacs [Isobe et al., 1995; Preston et al., 1995]. In the present study as well, most patients with mixed subtype infection had been at high risk for exposure to HCV. In these patients, superinfection (multiple infections) with HCV may play a role in inducing the immunologic environment, allowing the coexistence of multiple HCV subtypes and/or more HCV quasispecies.

Discrepancies in HCV subtype with different subtyping methods have been reported [Lau et al., 1995]. This discrepancy tends to be more frequent in patients with mixed (multiple) HCV infections such as hemophiliacs [Tuveri et al., 1997], and changes of the subtypes of infecting HCV have also been reported [Fujimura et al., 1996]. Two methods for subtyping were used in this study. Some patients found to have mixed subtype infections by these methods may be evaluated as patients with single subtype infection by another method. The existence of more HCV quasispecies, conversely, may affect these discrepancies and/or changes of HCV subtype according to the methods.

In conclusion, the number of HCV quasispecies was larger in patients with mixed HCV subtype infection than in patients with single subtype infection, suggesting that the immunologic environment influences coexistence of an increased number of HCV quasispecies in patients in whom HCV of multiple subtypes can exist. Conversely, particular environments may be necessary for coexistence of multiple HCV subtypes. Further studies will be required to reveal the details of the immunologic characteristics of patients in whom multiple HCV subtypes coexist.

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